2023

Hormonal modulation of disseminating endodontic infections

BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

HORMONAL MODULATION OF DISSEMINATING ENDODONTIC INFECTIONS

by

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Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Dentistry

In the Department of Translational Dental Medicine

2023

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DEDICATION

To my family who relentlessly believed in my potential and motivated me to realize it for myself. To my friends who have made my journey thus far immensely more meaningful and memorable. To my colleagues who have shared and endured the inevitable highs and lows along the way. And to my mentors who've generously poured their experiences, knowledge, and energy into shaping me into the person that I've always wished to become. *"If you want to go fast, go alone. If you want to go far, go together"*

ACKNOWLEDGMENTS

I want to thank Dr. Stashenko for his guidance and support throughout the entire project. His insight was irreplaceable and essential to my education at Boston University.

I would like thank Dr. Guoxian Wei for his expertise, consultation, and participation. Without him, this project would not have been possible.

I would like to thank Dr. Sasaki for providing the model design and structural template for this project. His advice throughout this project was critical.

I would like to thank Dr. Chogle for his support both spiritually and monetarily. His selflessness and generosity stabilized our team in the times we needed it most.

Lastly, I would like to thank my research confidante and future endodontist, Shimona Malik. You were everything I was not, and your dedication to this field will take you farther than the rest.

HORMONAL MODULATION OF DISSEMINATING ENDODONTIC INFECTIONS

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ABSTRACT

Objective: DEI sexual dimorphism has been observed where male but not female mice mildly immunosuppressed by blockade of IL-1 signaling, and challenged with an endodontic infection, developed facial abscesses, weight loss, splenomegaly, and sepsis which was often fatal. The central hypothesis is that estrogen increases the numbers and function of N1 neutrophils, resulting in effective anti- microbial immunity to DEI, whereas androgens are inhibitory. The aim of this study is to determine the effict of sex hormone modulation in protective immune responses to DEI and sepsis, specifically neutrophil-mediated resistance.

Materials and Methods: The therapeutic effects of estrogen and the androgen receptor antagonist enzalutamide (ENZ) on DEI will be evaluated in adult male group through direct observation of facial abscess formation, fatigue, and malaise. Additionally, survival

rates, weight change, and spleen weights will be recorded and compared between treatment groups. Male mice sub-groups will be categorized by hormonal treatments, which will be administered daily throughout a 31 day observation period after bilateral mandibular pulpal exposures and initiation of endodontic infections.

Results: Group 1 received no estrogen and no enzalutamide treatment following pulpal exposures. And 7 mice (n=7) were included in the experiment, however 1 mouse's final weight was unaccounted for due to the animal facility's onsite veterinarian sacrificing and removing the mouse during their daily heath assessment. Throughout the course of the 31 day experimental timeline, average weight loss of this group was 4.72 grams. The average final spleen weight was 0.11 grams. Group 2 received enzalutamide treatment only following pulpal exposures and had a total of 12 mice (n=12). 2 mice were observed to have developed facial abscesses over the course of the 31 day hormone treatment period, and 8/12 survived to the end of the experimental period. Throughout the course of the 31 day experimental timeline, the average weight loss of this group was 3.63 grams. The average final spleen weight was 0.11 grams. Group 3 received estrogen treatment only following pulpal exposures and had a total of 8 mice (n=8). 2 mice did not survive the pulpal exposure procedure and accounted for the only mice that were lost in this group resulting in 6/8 mice surviving through the experimental period. Throughout the course of the 31 day experimental timeline, the average weight loss of this group was 3.66 grams. The average final spleen weight was 0.083 grams. Group 4 received both estrogen and enzalutamide treatment simultaneously, totaling in 11 mice (n=11).

Throughout the experimental period, only 1 mouse was found deceased following the first round of hormonal treatments. Throughout the course of the 31 day experimental timeline, the average weight loss of this group was 1.45 grams. The average final spleen weight was 0.078 grams.

Conclusion: Estrogen's (E2) has a protective role on immune cells and function against DEIs, while enzalutamide (ENZ) appears to effect protection minimally. Based on the comparisons between weight changes, spleen weights, and survival rates, a combination of E2 and ENZ resulted in the least overall weight loss and spleen weights throughout the course of the experiment while the groups that received no treatment and only ENZ resulted in the highest average weight loss and spleen weights.

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LIST OF ABBREVIATIONS

AR ..... Androgen Receptor

DMSO ..... Dimethylsulfoxide

DEI ..... Disseminating Endodontic Infection

E2 ..... 17-β estradiol

ENZ ..... Enzalutimide

ER ..... Estrogen Receptor

GPER ..... G protein-coupled estrogen receptor

IFN ..... Interferon

IL ..... Interleukin

NFκB ..... Nuclear Factor Kappa B

NK ..... Natural Killer

OVX ..... Ovariectomized

PGG ..... Poly-[1-6]--D-glucopyranosyl-[1-3]--D-glucopyranose

PMNL ..... Polymorphonuclear Leukocyte

PMN ..... Polymorphonucleocyte

ROS ..... Reactive Oxygen Species

SCID ..... Severe Combined Immunodeficient

TNF ..... Tumor Necrosis Factor

CHAPTER 1: INTRODUCTION

Endodontic (dental pulp) infections are caused by a range of physical and biological insults, most commonly by dental caries, which progresses to involve the pulp. Physical trauma and thermal damage induce pulpal inflammation, which in turn facilitates the localization of blood-borne bacteria to pulpal tissue via the process of *anachoresis* (1). Pulpal infections elicit local immune responses, but these are rarely if ever able to clear the infection, which inevitably progresses to total pulp necrosis (1). This chronic infection induces a periapical immune response ('periapical lesion') with the resorption of alveolar bone detectable as a radiolucency.

The composition of the carious bacterial biofilm changes as infections progress through the layers of the tooth, particularly as the environment becomes more anaerobic. Pulpal infections are known to be polymicrobial, high in bacterial diversity, and predominantly anaerobic once the pulp vasculature is destroyed (2). In the majority of cases, these infections remain localized to the root canal system by the periapical immune response, which includes both innate (neutrophils, macrophages, NK cells) and adaptive immune cells (T and B lymphocytes and their subsets) (1). However, highly pathogenic combinations of infecting bacteria, combined with lowered host immunity may lead to infection dissemination, with the formation of an acute apical abscess, severe pain and swelling (3). If left uninhibited, these infections are able to spread systemically, resulting in sepsis and even death (4).

In these severe cases, epidemiologic studies have revealed the length of a hospital stay for patients with dentoalveolar infections is strongly correlated with underlying medical conditions, especially those that compromise the host's immune system (5). While the frequency of hospitalizations from periapical abscesses are relatively low, an eight-year study indicates a 41% increase in these hospitalizations and a 32% increase in emergency room visits.

Review of Literature:

1.1 Disseminating Endodontic Infections Pathophysiology

Mechanistically, the rapid progression of this infection leads to pulp cell and tissue death due to release of bacterial toxins and increased competition for nutrients (6). Immune cells, that are present in normal healthy pulp, such as dendritic cells and mast cells, serve as sentinels orchestrating the initial local immune response (7). The earliest responders include neutrophils and macrophages, followed by a significant increase in T and B lymphocytes and plasma cells at the site of bacterial invasion (8). Immune cells migrate to the site of infection via a process known as chemotaxis, followed by the release of metalloproteinases, reactive oxygen species (ROS), and other potent enzymes are released by the immune cells to combat the bacteria (7). Significant collateral damage to host tissue occurs as a result, releasing signals from damaged host cells, which exacerbates this proinflammatory response (9). The ultimate result is inevitably total pulp destruction and the presence of a chronic multi-species infection within the tooth, that cannot be resolved without root canal treatment.

If the pulpal infection is unable to controlled and localized to the tooth, an odontogenic abscess can form and ultimately disseminate systemically (10). Previous studies by Stashenko et al, investigated which immune cells and reactions are involved in preventing these disseminating endodontic infections. Initial studies compared RAG-2 severe combined immunodeficient (SCID) knockout mice to immunocompetent mice following surgical pulp exposure (11). This study concluded that mice lacking both B- and T-cell-mediated immunity developed disseminating infections (11). A follow up study compared B, T and complement-deficient mice to RAG-2 SCID, and found that mice lacking B cells were susceptible to dissemination, whereas T cell and complement deficient mice were not. Since the primary function of B cells is to differentiate into antibody-producing plasma cells,

Passive antibody transfer studies showed that anti-bacterial antibody-mediated mechanisms, most likely bacterial opsonization to facilitate phagocytosis and killing by neutrophils and macrophages, is essential to localizing anaerobic endodontic infections and preventing their systemic spread (12).

Components of the innate immune system play a critical role in controlling pulpal infections locally. A study by Stashenko et al. used PGG glucan, a biological response modifier, which stimulates neutrophil production and phagocytic/bactericidal activity in rats. After surgical pulp exposures and bacterial challenge from the oral environment, animals treated with PGG glucan exhibited significantly less infection stimulated periapical bone resorption and slower progression of radicular pulpal necrosis compared to untreated rats (13). It is apparent from these previous studies that B-cell antibody

mediated bacterial opsonization combined with neutrophil mediated phagocytosis are critical elements for protection against disseminating endodontic infections.

1.2 Neutrophils

The initial response to dental infections occurs locally by cells within the pulp tissue. The host's ability to control the infection at this stage is critical in preventing dissemination of endodontic infections. Neutrophils are known as one of the first responders to infection. They are categorized as polymorphonuclear leukocytes (PMNL), are the predominant immune cell population in human blood. Hematopoietic stem cells in the bone marrow undergo granulopoiesis and mature into neutrophils that then circulate throughout the body serving as the primary immune cell of the innate immune system (14). While neutrophils are produced in large numbers of approximately 10^{11} cells per day, they have a short life span and are constantly being replenished.

'Priming' or activation of cells refers to an external stimulus reaching the cell, which initiates a cellular response stimulating a transition from G0 to G1 (15). When neutrophils are primed, their safety mechanisms are unlocked and are allowed to activate completely. Primed neutrophils demonstrate increased phagocytic activity, increased cell migration, increased chemotaxis, increased myeloperoxidase activity, elevated oxidative metabolism, enhanced microbiocidal activity, enhanced cytotoxicity, and release of lysosomal enzymes (16). IL-1 has been shown to prime neutrophils for enhanced superoxide release in a dose-dependent manner (15). This process is achieved by both IL-

1 isoforms for human neutrophils through the MKK3/6-p38 MAPK cascade (17). Through previous studies, a relationship between sex hormones, IL-1 isoforms, and PMNs, resulting in sexual dimorphism in resistance to dental infections. However, further investigation is required to establish whether estrogen modulates IL-1 in priming immune cells.

1.2 Neutrophil Polarization: 'N1' and 'N2'

Recent data has revealed that neutrophils can differentiate or 'polarizing' into two distinct phenotypes, 'N1' and 'N2', in response to different environmental signals (18). Since Fridlender et al.'s (2009) initial discovery of neutrophil polarization in the presence of tumors, polarization of neutrophils have been associated with other inflammatory diseases as well (19). The 'N1' phenotype has been associated with pro-inflammatory microenvironments, whereas the 'N2' phenotype has been associated with anti-inflammatory microenvironments. The exact mechanism of neutrophil polarization is not fully understood, however, the data from previous studies have consistently suggested that the 'microenvironment' serves as highly influential to the induction of neutrophil polarization (18). Zhao et al.'s study investigated which environmental factors are associated with each phenotype and discovered that LPS + IFN-γ is the most commonly selected stimulator for N1 polarization, while IL-4 induced N2 polarization of neutrophils (18). The presence of N1 or N2 neutrophils could potentially give us insight into local

inflammatory environments and infection dissemination, as there are no present data on neutrophil polarization and bacterial infection.

1.3 Interleukin-1

Interleukin 1 (IL-1) has been demonstrated to prime human neutrophils for enhanced release of superoxide (O^2-), and triggered O^2- release in a dose-dependent manner (21). Additionally, Human recombinant IL-1 was also shown to prime PMNs for migration towards bacterial peptide chemotactins in an *in vitro* model (20).

Interleukin 1 (IL-1) (22) is generally considered as a prototypical pro-inflammatory cytokine (23), however its effects are not limited to inflammation. IL-1 has been associated with the inhibition of bone formation and activation of resorption (21). There are two distinct isoforms of IL-1; IL-1α and IL-1β. These isoforms exert their effects by binding to specific receptors; IL-1RI and IL-1RII (24). IL-1RI has a 213 aa cytoplasmic region, while IL-1 RII has only a short 29 aa cytoplasmic tail. This difference is reflected in the purported function of the IL-1RI as a signaling receptor, whereas IL-1RII appears to be an activity down-modulating 'dummy' receptor (21). While there are many reports on the two IL-1 receptors, the current evidence more strongly supports the concept of a single signaling receptor (IL-1RI) that is activated and preferentially binds to both isoforms of IL-1 (25,26). Previous studies on the role of IL-1 in DEI's indicates that it plays a protective role, suggesting the significance of IL-1 in localizing pulpal infections and preventing systemic spread and generalized sepsis (21). The results of this study on

neutralized IL-1 in mice, interestingly revealed a marked difference in the systemic dissemination of pulpal infections between males and females, with males exhibiting susceptibility and females being resistant. Considering these data, the role of sex hormones in disseminating endodontic infections warrants further investigation.

1.4 Estrogen Modulation of Immunity

Estrogens are a group of related steroid hormones comprised of a four-ringed fat molecule with a carbon backbone or core, similar to their cholesterol precursor. Various enzymes add or remove groups from the cholesterol core and thus, transform its beginning structure into the steroid pregnenolone, and then into androgens. Specific aromatase enzymes convert androgens into three forms of estrogen: estrone (E1), 17-β estradiol (E2), and estriol (E3). In non-pregnant women, 17-β estradiol is the most potent and predominant form of estrogen, while in post-menopausal women, estrone is most prevalent. During pregnancy, estriol (E3) becomes estrogen's primary form (36).

Sex hormones have a wide variety of biological responses, however, for the purpose of this study the focus is on how they affect host immunity. When considering the role of estrogen, mainly E2, in the context of infection, both human and non-human animal studies have demonstrated that males are more susceptible to bacterial, viral, and other types of infections (37). When considering estrogen's effect on host immunity, its circulating concentrations may dramatically affect infection resistance versus susceptibility, including the ability to localize and clear infections.

Estrogens generally enhance both cell-mediated and humoral immune responses; however, there have been reports of estrogen suppressing some cell-mediated responses (38). In a study comparing female mice treated with 17-β estradiol (E2) and untreated females and males, E2 treatment was reported to increase susceptibility to infections with *Listeria monocytogenes, Salmonella typhimurium, Toxoplasma gondii*, and disseminated gonococcal infection (39,40). It is important to consider not only estrogen's role on host immunity, but also which immune cells are being modulated. In these studies, the data suggest that increased susceptibility to these infections in estrogen-treated mice may be due to reduced innate immunity (i.e. NK cell and macrophage activation) and lower pro-inflammatory cytokine responses (e.g. IL-2 and TNFα production) (41).

1.5 Estrogen and Immune Cells

Estrogen's ability to influence immune cells depends not only on its circulating concentrations but also on the availability and affinity of target-tissue receptors (42). Estrogen receptors are required for the hormone's interaction with immune cells. Studies have identified various lymphoid organs including the thymus, bone marrow, and spleen, in rodent and human macrophages, circulating CD8+ T-cells and splenocytes to have high-affinity estrogen as well as androgen receptors (43,44,45). However, there are no current studies that have examined whether changes in the expression of sex steroid receptors mediate the effects of estrogens and androgens on infection.

Classic studies have established estrogen's ability to exert its action on immune cells through two types of nuclear receptors; ER-alpha and ER-beta (46). The mechanism of both receptors involves ligand binding to surface membrane receptors, dimerization, internalization and binding to specific response elements in the target genes to elicit a transcriptional response (46). Styger et al (2007) identified estrogen receptors on polymorphonuclear and mononuclear leukocytes in peripheral blood in both males and females (47). A third estrogen receptor, known as G protein-coupled receptor-1 (GPER), has been identified and described by Prossnitz et al., 2009 (48). GPER is classified as a member of the G protein-coupled receptor superfamily, which contains seven transmembrane helices and mediates estrogen dependent kinase activation and transcriptional responses.

1.6 Estrogen and Neutrophils

Data on estrogen's effect on neutrophils are somewhat contradictory, as some studies have observed an increase in neutrophil numbers in cancer patients that received estramustine phosphate, a chemotherapic drug that elevates serum 17-β estradiol levels. However, there are other data that estrogen has a suppressive effect on numbers of circulating lymphocytes, neutrophils, and monocytes in ovariectomized mice (49,50). One hypothesis regarding these findings is that estrogen stimulates splenic lymphoid cells to secrete neutrophil attracting chemokines or promotes the production of neutrophil-attracting chemokine such as MCP-1 in splenocytes (51), or that estrogen delays

spontaneous neutrophil apoptosis (52). Further investigation on the mechanism underlying estrogen's influence on immunity is needed.

1.7 Testosterone Regulation of Immune Function:

Concomitant neutralization of both IL-1α and IL-1β resulted in dramatically increased susceptibility of male but not female mice to disseminating bacterial pulpal infections (53). Similarly, male but not female mice with genetic knockout of the IL-1RI were also susceptible, demonstrating a clear sexual dimorphism with regard to the ability to localize these infections to the root canal (53,60). Ovariectomized (OVX) female mice developed disseminating infections at a similar rate to male mice, which was reversed by an estrogen implant (53). This finding is consistent with the general notion that estrogen induces protective immunity, whereas male sex hormones may be immunosuppressive (54,55,56).

Testosterone, the predominant sex hormone in males, has been reported to reduce natural killer (NK) cell activity in mice, reduce synthesis of proinflammatory cytokines, including TNFα through the inhibition of transcriptional factors such as nuclear factor kappa B (NFκB) (57,58). While studies have shown a positive correlation between male subjects and immunosuppression, it is yet to be determined if sex differences in immune function are a result of direct hormonal action or other possible interactions among additional hormones (e.g. androgens, glucocorticoids, prolactin, or melatonin) resulting in these findings (56).

To investigate testosterone's influence on immunomodulation of disseminating bacterial infections, we will treat male mice with enzalutamide (ENZ), an androgen receptor (AR) signaling inhibitor. ENZ has been successfully used to inhibit AR signaling, especially in the lack of AR agonist activity (59).

1.8 Murine Model of Disseminating Dentoalveolar Infection

When considering which specific immune responses are critical for the prevention of the spread of endodontic infections, it is understood that the earliest pulpal and periapical responses to bacterial invasion and/or the diffusion of bacterial products through dentinal tubules include the influx of polymorphonuclear leukocytes (PMNs) and monocytes (53,69). With more persistent infection the response becomes mixed with infiltration of T cells, B cells, and plasma cells in addition to the earlier migrating phagocytes (61,62).

It is well-established that disseminating endodontic infections are almost exclusively caused by polymicrobial anaerobic bacteria (65). Previous studies investigating dentoalveolar infections have development and demonstrated an infection induction protocol paired with pulpal exposures in murine models that utilized a combination of 4 anaerobic bacteria for all previous studies, in order to standardize the infectious challenge in different mouse strains and experiments (2,9,53,60,69). Our current study will use the same 4 anaerobic pathogens, *including Prevotella intermedia, Fusobacterium nucleatum, Peptostreptococcus micros,* and *Streptococcus intermedius* in our current study (2).

Previous studies have shown that B cell-, but not T cell-deficient mice were susceptible to abscess formation and sepsis, and that passively transferred antibody, primarily IgG, against infecting bacteria was protective. This conclusion suggests that an antibody-mediated mechanism, most likely bacterial opsonization, is of importance in localizing anaerobic root canal infections and preventing their systemic spread. (5-6)

In another study, mice deficient in both P- and E-selectins, which mediate rolling adhesion of leukocytes to blood vessel walls, showed reduced PMN emigration, leading to increased bacterial tissue invasion, and decreased ability of the host to slow the progress of dentoalveolar infection (65,67,68).

Conversely, Sprague-Dawley rats treated with the immunomodulator, poly-(1-6)- beta-D-glucopryanosyl-(1-3)-beta-D-glucopyranose (PGG)-glucan, which stimulates neutrophil production and upregulates phagocytic and bactericidal activity, demonstrated greater resistance to infection, soft tissue destruction, and decreased pulpal necrosis (66).

Taken together, these data strongly indicate that antibody-mediated bacterial opsonization combined with efficient phagocytosis and killing of bacteria by neutrophils, are critical elements for protection.

The Stashenko Lab demonstrated that IL-1 possesses an important protective role in the early stages after pulpal infection (53). The lack of least one IL-1 isoform affects IL-12 and IFNγ production, which may negatively affect 'priming' leukocytes to efficiently fight local infections of the root canal system and prevent their systemic spread (53). The

estrogen effect on leukocytes, including induced IL-1 expression, may be a mechanism that explains the observed increased resistance of females vs. males to severe orofacial infections (53).

In the present study, we further investigated the role of phagocytic leukocyte priming in the context of sexual dimorphism / hormones, using a novel mouse model of disseminating dentoalveolar infections.

CHAPTER 2: Materials and Methods

2.1 Mice:

IL1-R1 knockout and c57Bl/6 male and female mice aged 8 to 28 weeks old were purchased from Jackson Laboratory, Bar Harbor, ME, USA (1). Animals were bred and maintained in a conventional environment in the BU animal laboratory, according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) under protocol #14-009. All procedures confirmed to the National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH publication no. 8023, 2010).

2.2 Bacterial Growth:

Four human dentoalveolar pathogens, Fusobacterium nucleatum ATCC 25586 Streptococcus intermedius ATCC 27335, Parvimonas micra formerly Micromonas micros and Peptostreptococcus micros 33270 and Prevotella intermedia ATCC 25611, were grown on plates of Brucella Blood Agar with Hemin and Vitamin K (Thomas Scientific, C838L74) under anaerobic conditions (80% N2, 10% H2, 10% CO2), harvested and suspend the bacterial cells in 1ml of PRAS The concentration of each cells were 3 x 10^9 cells/ml. Equal numbers of the four bacteria were mixed and methylcellulose at final concentration of 10mg/ml was added to the bacterial mix. Bacterial mix was then transferred from the anaerobic chamber in an sealed container with oxygen exposure indicators packets. Aliquots of (5ul) were transferred into the tooth

pulp chamber, instrumented down mesial and distal canals of each mice's first mandibular molars, and teeth were sealed with CAVIT (53).

Figure 1. Anaerobic Chamber used to culture multi-bacterial mixture that was introduced to exposed mouse pulp chambers for inducing endodontic infection.

2.3 Estrogen (E2) and Enzalutamide (ENZ) Supplementation:

17β- estradiol (MedChem Express) was dissolved in DMSO, and Enzalutamide (MedChem Express) was diluted in a mixture of Tween-80 and PEG-400. On day one, E2 was injected subcutaneously at around 50 µg/kg and ENZ was inserted orally using a

gavage at around 50 ug/kg. Hormone solutions batches were mixed on day of administration. The experimental groups were as follows: 8 mice treated with E2 only, 11 mice treated with ENZ only and 11 mice treated with E2 and ENZ. Each group was treated for 5 days per week for 3 weeks. The control group consisted of 7 mice that were not treated with any hormones.

2.4 Dentoalveolar infections

On day two, after the introduction of hormone treatment, the experimental mouse groups were introduced to the bacterial pathogens. For infection induction, mice were mounted on a jaw retraction board and anesthetized with a mixture of ketamine HCl (80 mg/kg) and xylazine (10 mg/kg) in 0.9% sterile saline by intraperitoneal injection. Both lower first molar dental pulps were exposed using a #1/4 round bur under a surgical microscope (Zeiss OPMI PICO S100). A mixture of the four human dentoalveolar pathogens were inoculated into the canals using a pipette and advanced apically into the canal using a size #6 K-file. Once the bacteria were introduced, the access cavity was sealed using 3M Cavit temporary filling material. All mice were weighed regularly and monitored for 4 weeks for the development of facial abscess or sepsis.

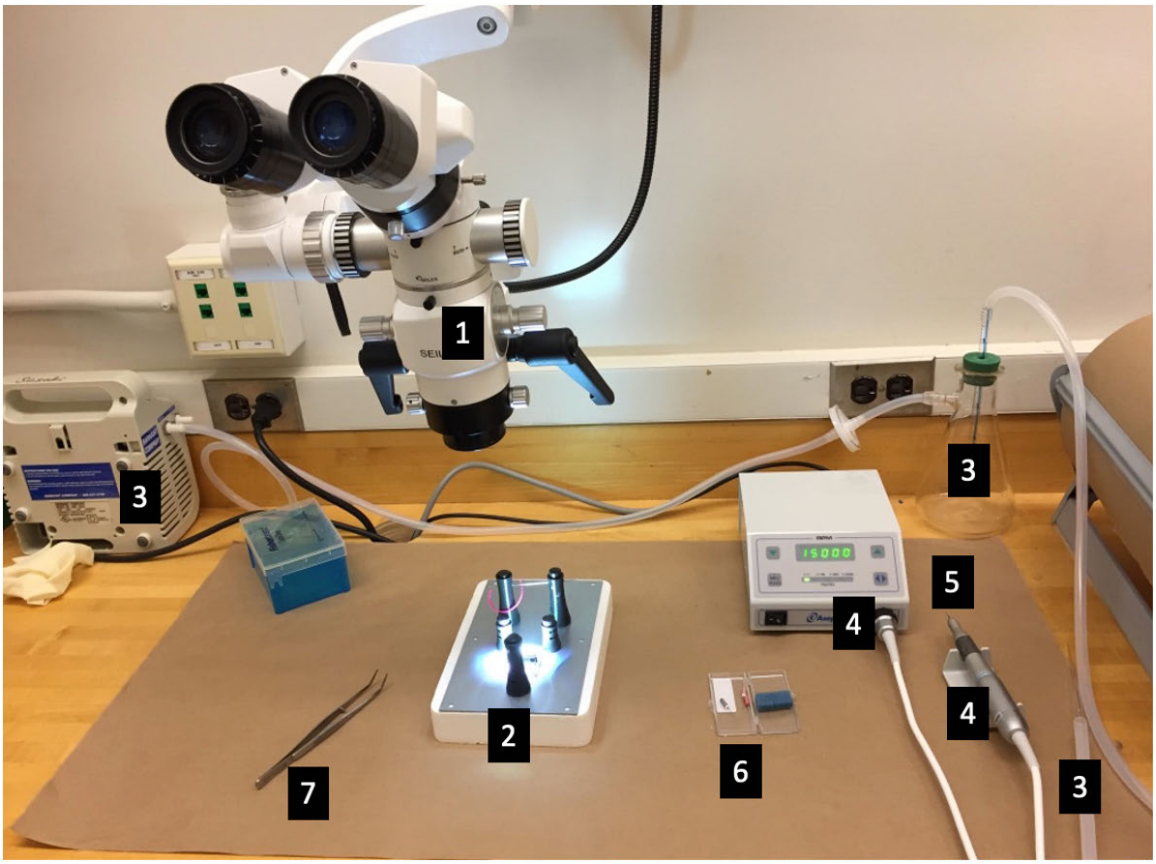

Figure 2. Surgical Set Up for Pulpal Exposure Procedure. 1) Zeiss OPMI PICO S100 Microscope. 2) Jaw Retraction Board. 3) Microsuction. 4) Slow Speed Endodontic Motor and Straight Handpiece. 5) #4 Surgical Round Bur. 6) #6 Endodontic C-file. 7) Cotton Pliers.

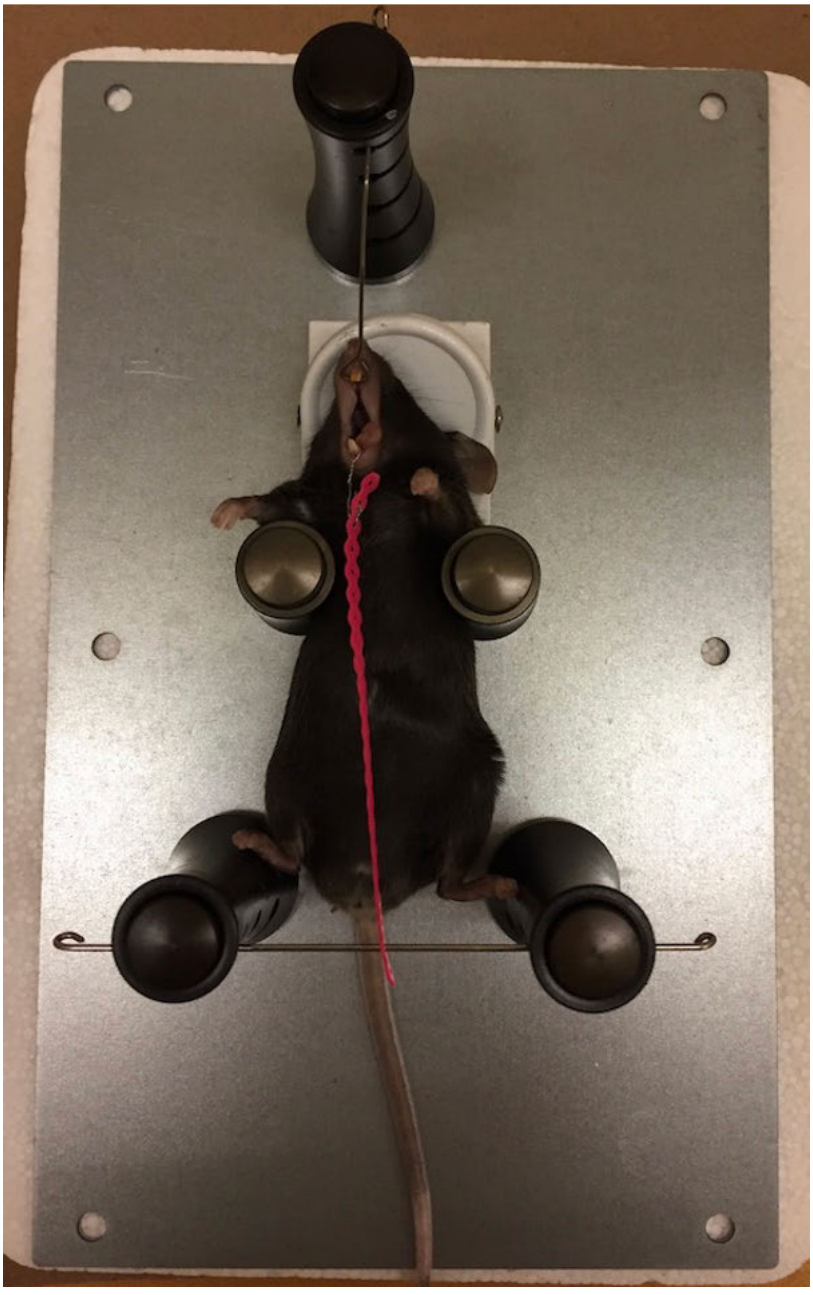

Figure 3. Mice retraction board used to stabilize anesthetized mice and retract jaw for pulpal exposure procedure.

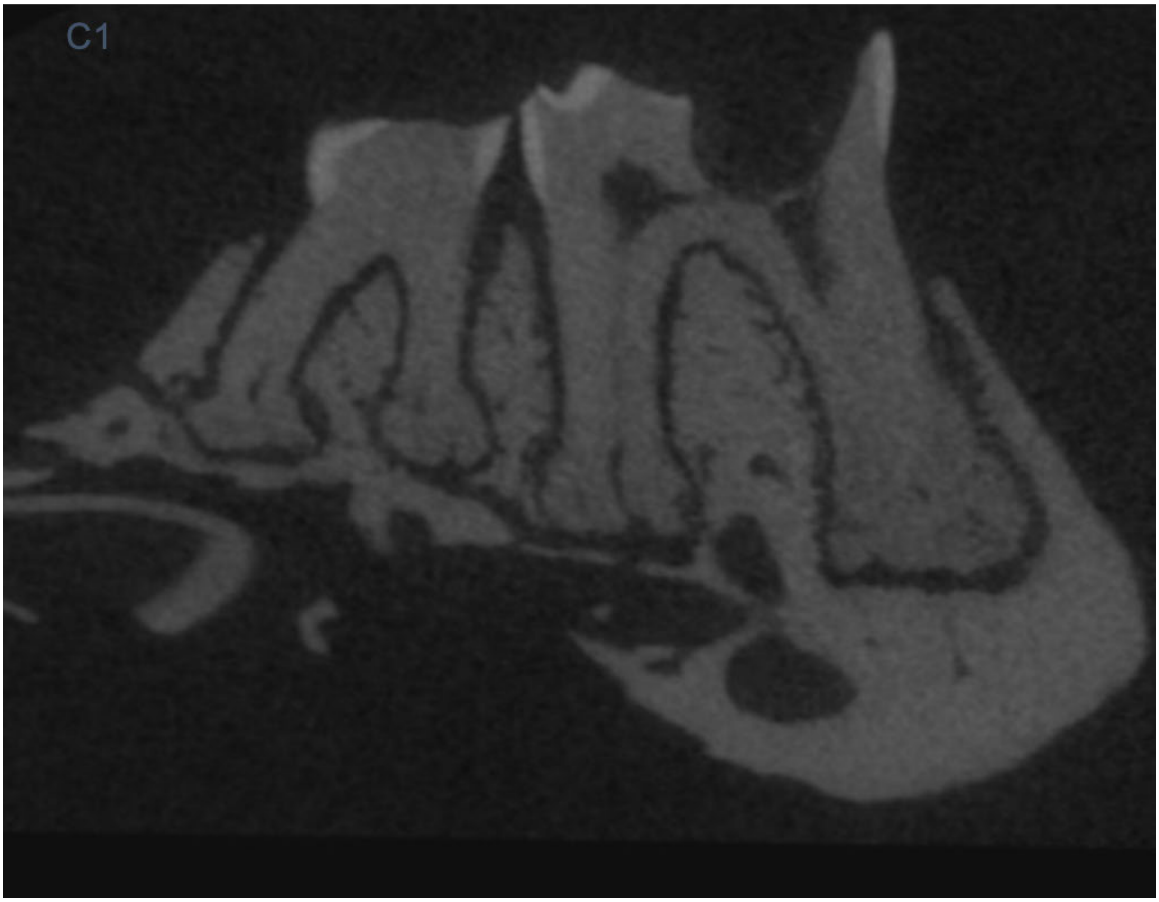

Figure 4. Micro-CT of Surgical Pulpal Exposure on murine mandible 1st molar

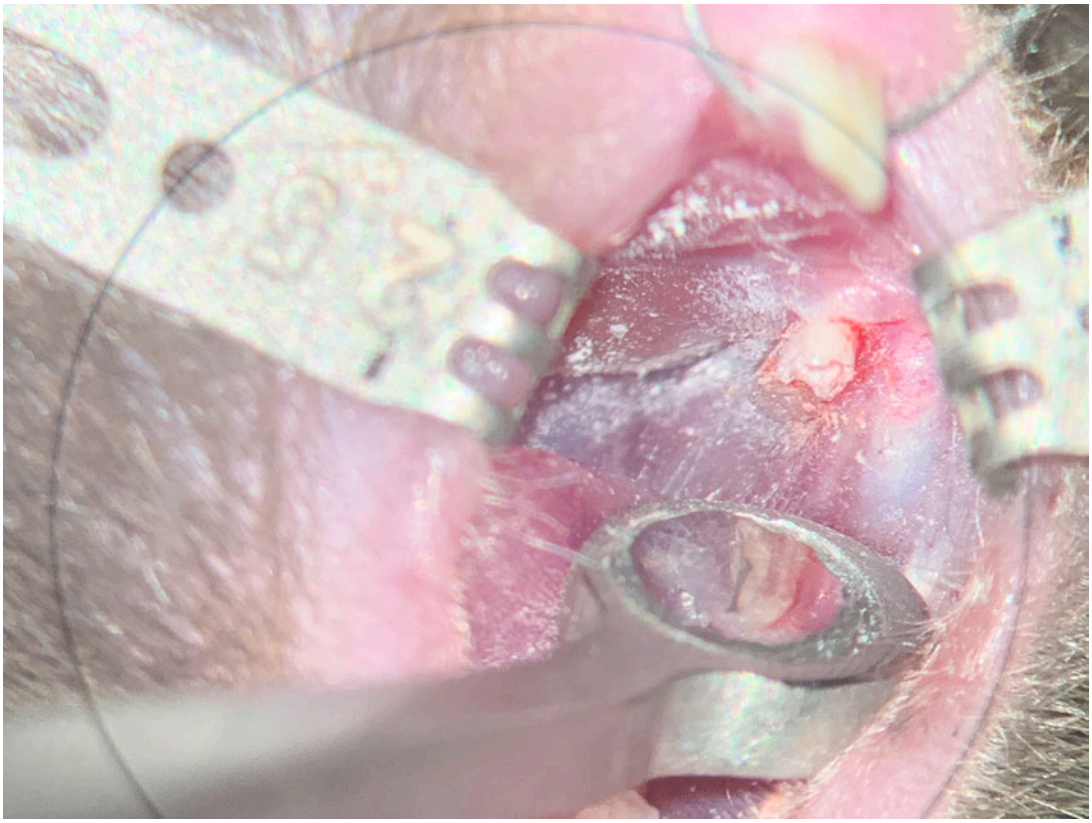

Figure 5. Mandibular 1st Mouse Molar following pulpal exposure procedure. Mesial and Distal canals visible for instrumentation with bacterial mixture.

2.5 Sample Preparation

After 4 weeks, granulation tissue and spleen samples were collected from animals immediately following sacrifice. Mice were euthanized using a CO2 gas chamber inhalation and spinal dislocation. The jaws were resected, and granulation tissue/surrounding hard and soft tissue was removed from the infected teeth using a 40x magnification surgical microscope. Separately, each animal's spleen was collected and

weighed. Both the granulation tissue and spleens were placed in RNA Protect (Qiagen) and stored in –80 C.

Figure 6. Unilateral dental abscess formation on Day 31 prior to sample collection.

Figure 7. Abscess exudate from dental abscess on sacrificed mouse subject during sample collection.

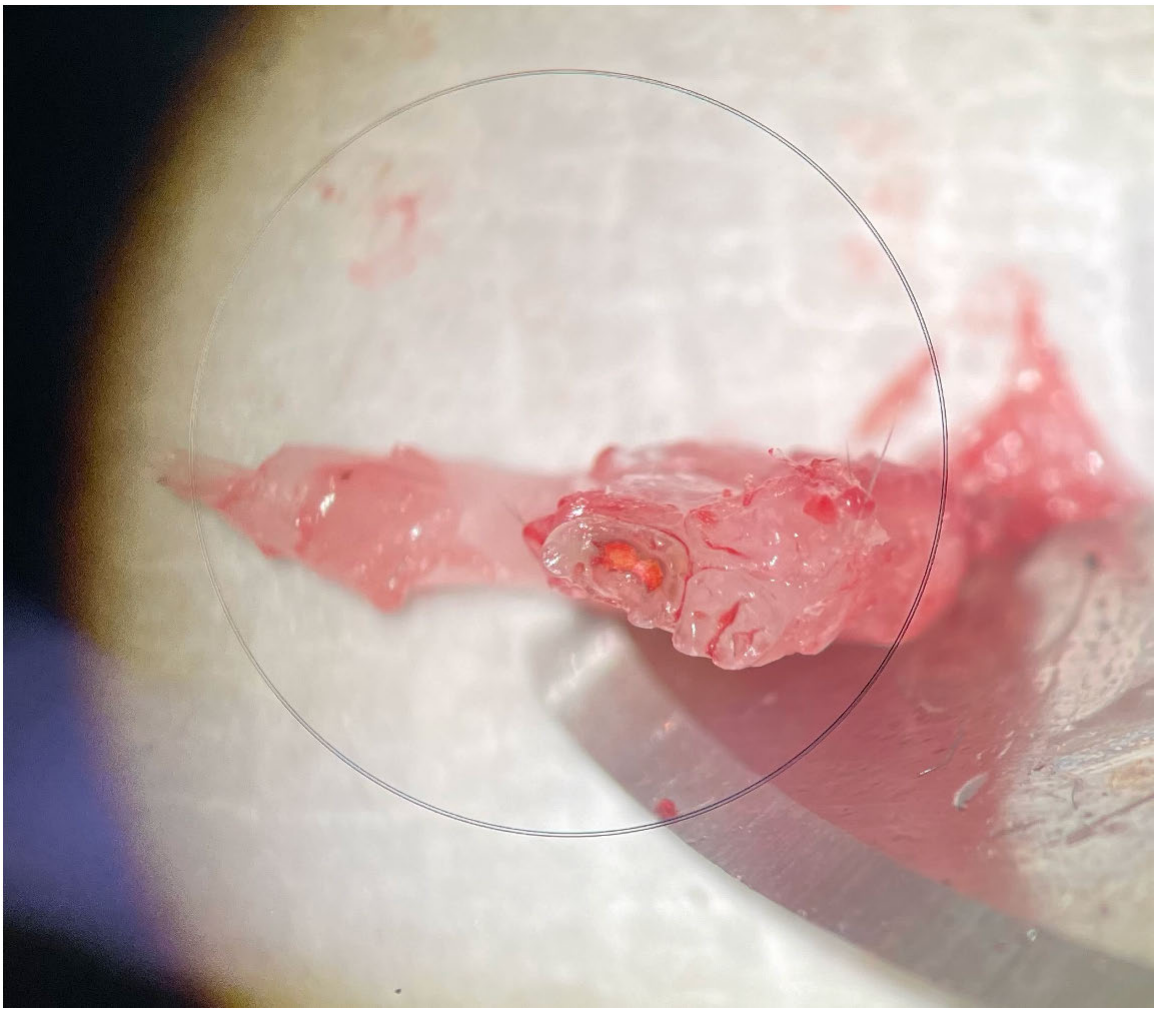

Figure 8. Hemisection of Mandible with tooth and granulation tissue from mouse jaw for sample collection.

2.6 RNA Isolation & Sequencing

Granulation tissue and spleen samples were lysed and homogenized using a homogenizer (Precellys 24). Purification steps from Qiagen MicroRNA kits (Cat. No. 217084) were followed to isolate RNA. After isolation, a NanoDrop ND-1000 UV-Vis Spectrophotometer was used to determine the concentration of purified RNA.

16 purified RNA samples from granulation and teeth samples were homogenized and stored in -80 C conditions in Qiazol liquid. Acid washed stainless steel beads were added to thawed samples and homogenized in Qiagen TissueLyser II for 2 minutes. Samples were then processed by Boston University's Microarray and Sequencing Resource Core Facility for RNA sequencing analysis.

Figure 9. Sterilized stainless steel beads used for homogenization of samples. Four samples collected from each of the four hormone treatment groups.

Figure 10. Tissue Lyser II used to homogenize tissue samples prior to analysis.

CHAPTER 3: Results

3.1 Mouse Weight Change:

The mean weight change amongst the four treatment groups ranged from 1.45g to 4.71g. Statistical analysis using one-way analysis of variance (ANOVA) to determine the existence of the difference in the obtained group means. In the test, the hormonal treatment was considered a factor and the change in weight over the treatment period was considered the dependent variable. The test determined that there was no statistical difference amongst the 4 groups, indicated by a p-value of 0.6643 with the alpha set as 0.05. Therefore, the null hypothesis cannot be rejected. The data, however, can be interpreted as a trend according to expected results. The group that showed the largest change in weight over the treatment period of 4.71g lost, was the group that received no hormonal treatment. The treatment group that received both E2 and ENZ treatment, showed the least mean weight loss across the group at 1.45g lost over the treatment period.

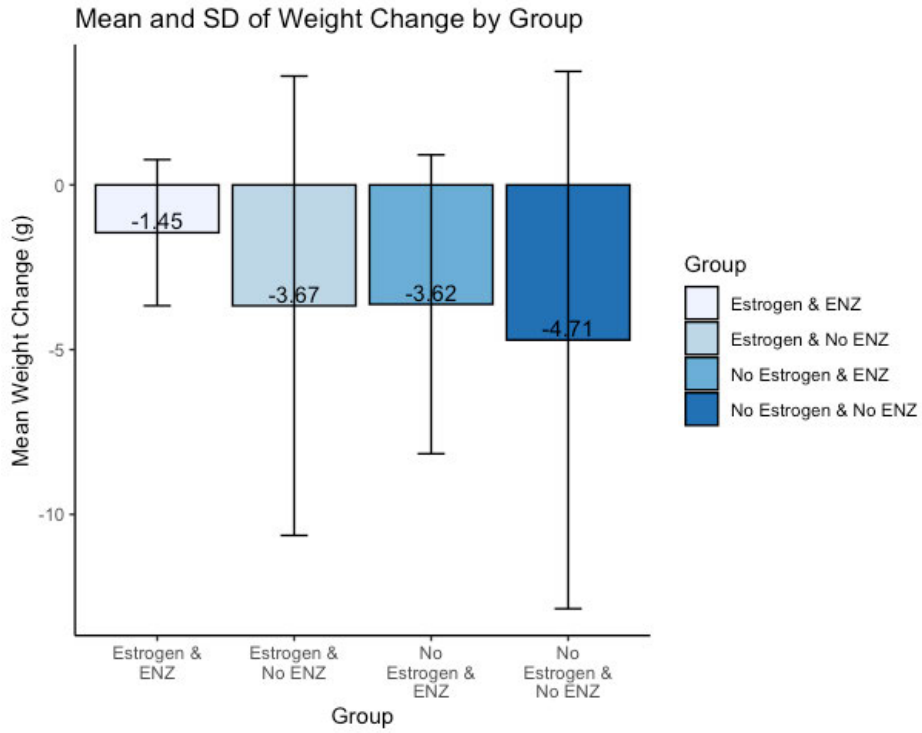

Figure 11. Graph of Mean Weight Loss for four treatment groups.

Figure 12. Histogram of Weight Change for four treatment groups.

ANOVA for Weight Change by Group

	Estimate	Std. Error	T value	Pr(>|t|)
Intercept	-1.450	1.714	-0.846	0.405
Estrogen & No ENZ	-2.218	2.798	-0.793	0.435
No Estrogen & ENZ	-2.175	2.570	-0.846	0.405
No Estrogen & No ENZ	-3.258	2.798	-1.164	0.255

	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Group	3	46.87	15.623	0.5321	0.6643
Residuals	26	763.39	29.361		

Table 1. ANOVA for Mean Weight Change for each of the four groups; E2 and ENZ treatment group, E2 only treatment group, ENZ only treatment group, and No treatment group.

3.2 Spleen Weights:

The mean spleen weight amongst the four treatment groups ranged from 0.08g to 0.11g. Statistical analysis using one-way analysis of variance (ANOVA) to determine the existence of the difference in the obtained group means. In the test, the hormonal treatment was considered a factor and the final spleen weight was considered the dependent variable. The test determined that there was no statistical difference amongst the 4 groups, indicated by a p-value of 0.3067 with the alpha set as 0.05. Therefore, the null hypothesis cannot be rejected. The data, however, can also be interpreted as a trend according to expected results. The group that showed the highest spleen weight 0.11g, were the groups that did not receive E2 treatment. The treatment group that received both E2 and ENZ treatment and the E2 treatment only group, showed the lowest final mean spleen weight across the groups at 0.08g.

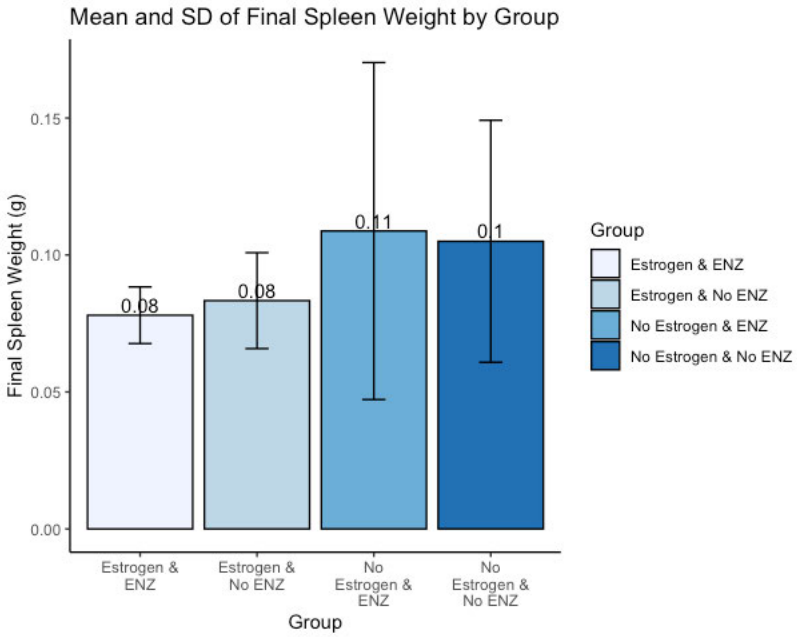

Figure 13. Graph of Mean Final Spleen Weights for four treatment groups.

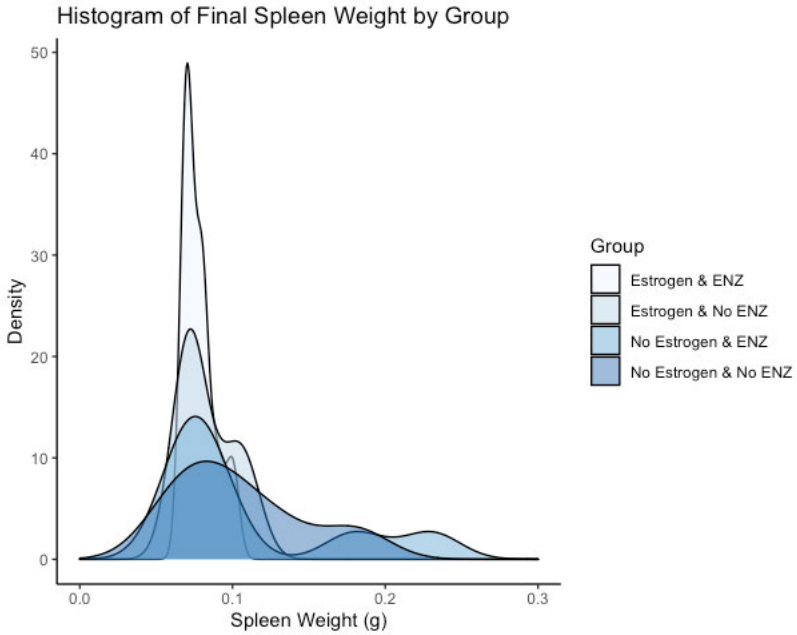

Figure 14. Histogram of Final Spleen Weights for four treatment groups.

ANOVA for Final Spleen Weight by Group

	Estimate	Std. Error	T value	Pr(>|t|)
Intercept	0.0780	0.0122	6.391	9.05e-07
Estrogen & No ENZ	0.0053	0.0199	0.268	0.791
No Estrogen & ENZ	0.0308	0.0183	1.680	0.105
No Estrogen & No ENZ	0.0270	0.0199	1.355	0.187

	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Group	3	0.0057	0.0019	1.2656	0.3067
Residuals	26	0.0387	0.0015		

Table 2. ANOVA for Mean Spleen Weight for each of the four groups; E2 and ENZ treatment group, E2 only treatment group, ENZ only treatment group, and No treatment group.

CHAPTER 4: Discussion

The purpose of this study was to further investigate the role and underlying mechanism of sex hormones in the context of modulation of the immune response to disseminating endodontic infections (DEI). A previous study on DEI investigated the role of IL-1, concluding that IL-1 plays an important protective role in the early stages after pulpal infection (53). While that study established that IL-1 signaling is critical to the ability of the host to localize infections to the tooth, the data further demonstrated that the lack of signaling affected male mice more profoundly than female mice (53). From this observation, estrogen was suspected to have a protective role against DEI, which was confirmed by the findings that ovariectomy resulted in female susceptibility to DEI, an effect that was reversed by estrogen supplementation (53). The protective function of

estrogen against DEI is consistent with other studies investigating sexual dimorphism in the immune response to infections (38, 81,82,83,84). A consistent finding from such work strongly indicates that estrogen is protective, whereas testosterone is generally immunosuppressive (85,86,87).

In the present study, we sought to extend this concept to determine if male mice with blocked IL-1 signaling which are susceptible to DEI, could be protected by hormonal modulation. The interventions included the administration of estrogen, the testosterone receptor inhibitor enzalutamide, and a combination of both estrogen and enzalutamide. The parameters examined included the development of orofacial abscesses as an indicator of regional dissemination, weight loss and splenomegaly as manifestations of systemic spread of infection (sepsis).

In the previous study (53), it was found that immunocompromised males developed facial abscesses 5-7 days after infection, pathognomonic for DEI. Additionally, there was a stark difference between the male and the female mouse group in regards to indicators of infection dissemination including reduced physical activity, loss of body weight, and splenomegaly, all indicators of sepsis.

Sepsis resulting in multiple organ failure is largely attributed to endotoxins released during severe gram-negative bacterial infections and carries a staggering 30-50% mortality rate (71). Septic shock follows massive bacterial infections, resulting in the release of bacterial products and the activation of host immune cells and of soluble factors such as complement and clotting molecules. It is generally recognized that the severe pathology associated with septic shock results from a hyperactive and out-of-

balance network of endogenous pro-inflammatory cytokines, including TNF-α, IL-12, IL-6, and IFNγ (72). As a result, endotoxemic animals suffer from generalized intravascular coagulation with multiple organ failure as evidenced by severe congestion, hemorrhage, fibrin deposits, edema, thrombosis, massive accumulation of leukocytes in the lungs and the intestinal tract, severe congestion of the medullary sinusoids in the spleen, and segmental ischemia of the bowel with regions of hemorrhage or necrosis and an infracted caecum (73). Congestion of the spleen and impaired digestive function under septic conditions result in splenomegaly and weight loss.

The groups that exhibited the highest weight loss following induction of endodontic infections were the no treatment group followed by the group treated only with enzalutamide, which was ameliorated by hormonal treatment with estrogen and enzalutamide, along with a lesser effect of estrogen treatment alone. This trend is consistent with the findings from previous studies (53) and further supports the protective role of estrogen in immune function against DEI.

When comparing splenomegaly among the various treatment groups, we saw a similar trend, as the groups that received no treatment and enzalutamide only had the highest overall mean spleen weight, indicating the presence of blood borne infection. In contrast, the groups that received estrogen only and a combination of enzalutamide and estrogen showed lower average spleen weights. These findings further support a protective function of estrogen against DEI.

Splenomegaly in murine models investigating disseminating infections is an important clinical indicator, as the spleen is a critical peripheral immune organ (74).

While the exact mechanisms of splenomegaly resulting from systemic dissemination of infection are not completely understood, the spleen sits astride the vascular system and acts as a filtration system in which phagocytic cells, principally macrophages and neutrophils, phagocytize and kill circulating bacteria. There is a clear correlation between splenomegaly and septic infection, as is well established in various murine models (53,74,75,76,77).

In contrast to the previous study using this model (53) in which 30-50% of male mice died after IL-1 signaling blockade and pulpal infection, mortality rates were very low in this study. The only group that appeared to be significantly affected was the enzalutamide treated group, in which four mice died after infection and two mice were survived to the end of the observation period but had developed facial abscesses.

None of the other groups experienced significant post-infection mortality. This unexpected result was likely the consequence of utilizing IL-1R1 knockout animals in these studies, instead of the administration of anti-IL-1α and anti-IL-1β neutralizing antibody blockade. Our modification to the prior antibody-mediated inhibition of IL-1 ligands was proposed in order to increase the inhibition of IL-1 signaling by genetically removing the receptor completely, rather than relying on the action of antibodies for inhibition, which may be less profound and have left some receptors biologically functional. Unfortunately, the dramatic DEI phenotype was not observed in IL-1R1-/- male mice in these studies. Rather a much less dramatic sepsis was observed that was mainly manifested by trends toward loss of body weight, and splenomegaly, with frank facial abscesses and mortality rare events.

A phenomenon known as 'biological robustness' has recently emerged however, that may explain the divergence in phenotype between genetically-modified knockout mice and antibody neutralized mice. Biological robustness suggests that the loss of one gene may be compensated by another with overlapping function(s) and expression patterns (78), which has been reported by several gene knockouts in a range of model organisms (79,80). In essence, the ability to overcome the loss of certain genetically modified functions can be compensated by upregulation of other genes with similar functions. In the case of DEI, we have hypothesized that neutrophil activity is critical in limiting the spread of pulpal infections, by skewing the phenotype toward a pro-inflammatory N1 vs immunosuppressive N2 phenotype. It is entirely possible that other neutrophil priming mediators, including TNFα and possibly others, could compensate to induce N1 polarization in response to DEI. Compensation would have been operative from the time of birth in the IL-1R1 knockout mice, giving sufficient time to adapt to the loss of IL-1 signaling. In contrast, antibody neutralization is an acute intervention in animals with an intact IL-1 signaling system, in which there would be no time for compensation to occur after pulpal infection. This interpretation can in fact be tested in future studies, since we have collected and preserved spleen samples from all four treatment groups, and could evaluate gene expression by RNASeq to determine if mediators such as TNFα are upregulated, and if there are significant numbers of N1 vs N2 neutrophils, as determined by the expression of markers for these cells in the spleen.

In conclusion, the findings from this study further suggest estrogen's protective role in host immunity. Estrogen has the potential to be utilized as an adjunct therapy against

disseminating endodontic infections in the future, however, additional investigation is necessary in order to further understand the mechanisms in which estrogen boost immune response. Additionally, our novel murine model introduces speculation into future studies due to the concept of 'genetic robustness'. Understanding compensatory actions against breeding genetically immunodeficient animal subjects is essential in producing applicable and reliable data.

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